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(54) **Novel coleopteran-active bacillus thuringiensis isolate and a novel gene encoding a coleopteran-active toxin.**

(57) The subject invention concerns a novel microbe and gene encoding a novel toxin protein with activity against insect pests of the order Coleoptera. Pests in the order Coleoptera do heavy damage to crops, e.g., corn. The novel Bacillus thuringiensis microbe of the invention is referred to as B.t. PS50C. The spores or crystals of this microbe, or mutants thereof, are useful to control coleopteran pests in various environments. The novel gene of the invention can be used to transform various hosts wherein the novel toxic protein can be expressed.

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Background of the Invention

Bacillus thyringiensis (B.t.) produces an insect toxin designated as δ-endotoxin. It is synthesized by the B.t. sporulating cell. The toxin, upon being ingested in its crystalline form by susceptible insect larvae, is transformed into biologically active moieties by the insect gut juice proteases. The primary target is insect cells of the gut epithelium, which are rapidly destroyed.

The reported activity spectrum of B.t. covers insect species within the order Lepidoptera, many of which are major pests in agriculture and forestry. The activity spectrum also includes the insect order Diptera, which includes mosquitos and black flies. See Couch, T.L. (1980) "Mosquito Pathogenicity of Bacillus thuringiensis var. israelensis." Developments in Industrial Microbiology 22:61-76; Beegle, C.C., (1978) "Use of Entomogenous Bacteria in Agroecosystems," Developments in Industrial Microbiology 20:97-104. Krieg, et al., Z. ang. Ent. (1983) 96:500-508, describe a B.t. isolate named Bacillus thuringiensis var. tenebrionis, which is reportedly active against two beetles in the oder Coleoptera. These are the Colorado potato beetle, Leptinotarsa decemlineata, and Agelastica alni.

In European Patent Application 0 202 739 there is disclosed a novel B.t. isolate active against Coleoptera. It is known as B. thuringiensis var. san diego(B.t.sd.). U.S. Patent No. 4,966,765 discloses the coleopteran-active Bacillus thuringiensis isolate B.t. PS86B1. European Patent Application 0 337 604 also discloses a novel B.t. isolate active against Coleoptera. This isolate is B.t. PS43F.

Coleopteran-active strains, such as B.t.sd., B.t. PS86B1, and B.t. PS43F, can be used to control foliar-feeding beetles. The Colorado potato beetle (Leptinotarsa decemlineata), for example, is susceptible to the delta-endotoxin of B.t.sd. and larvae are killed upon ingesting a sufficient dose of spore/crystal preparation on treated foliage.

A number of crops are attacked by flea beetles. These beetles belong to the family Chrysomelidae, the decemlineata. The adults can cause extensive damage by feeding on the foliage.

Brief Summary of the Invention

The subject invention concerns a novel Bacillus thuringiensis (B.t.) isolate and a cloned gene therefrom which encodes a novel coleopteran-active protein. The novel B.t. isolate, known herein as Bacillus thuringiensis PS50C (B.t. PS50C), has thus far been shown to be active against the Colorado potato beetle (Leptinotarsa decemlineata). The novel δ-endotoxin gene of the invention encodes an ≈130 kDa protein. The nucleotide sequence of the gene (open reading frame only) is shown in Sequence ID No. 1. The predicted peptide sequence of the toxin is shown in Sequence ID No. 2.

The subject invention also includes mutants of B.t. PS50C which have substantially the same pesticidal properties as B.t. PS50C. Procedures for making mutants are well known in the microbiological art. Ultraviolet light and nitrosoguanidine are used extensively toward this end.

Further, the invention also includes the treatment of substantially intact B.t. PS50C cells, and recombinant cells containing the gene of the invention, to prolong the pesticidal activity when the substantially intact cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or a combination of chemical or physical means, so long as the technique does not deleteriously affect the properties of the pesticide, nor diminish the cellular capability in protecting the pesticide. The treated cell acts as a protective coating for the pesticidal toxin. The toxin becomes available to act as such upon ingestion by a target insect.

Detailed Disclosure of the Invention

The novel Bacillus thuringiensis isolate of the subject invention has the following characteristics in its biologically pure form:

Characteristics of *B.t.* PS50C

Colony morphology--Large colony, dull surface, typical B.t.

Vegetative cell morphology--typical B.t.

Culture methods--typical for B.t.

Flagellar serotyping--PS50C belongs to serotype 18, kumamotoensis.

Crystal morphology--a sphere.

RFLP analys--Southern hydridization of total DNA distinguishes B.t. PS50C from B.t.sd. and other B.t. isolates.

Alkali-solubl proteins--SDS polyacrylamide gel electrophoresis (SDS-PAGE) shows a 130 kDa doubl t protein.

A comparison of the characteristics of B. thuringiensis PS50C (B.t. PS50C) to the characteristics of th known B.t. strains B. thuringiensi var. san diego (B.t.sd.), B. thuringiensis PS86B1 (NRRL B-18299), and B. thuringiensis var. kurstaki (HD-1) is shown in Table 1.

Table 1. Comparison of B.t. PS50C, B.t. PS86B1, B.t.sd., and B.t. HD-1

	B.t. PS50C	B.t.sd.	B.t. PS86B1	B.t. HD-1
Serovar	kumamotoensis	morrisoni	tolworthi	kurstaki
Type of inclusion	sphere	square wafer	flat, pointed ellipse, plus sm. inclusions	Bipyramid
Size of alkali-soluble proteins by SDS-PAGE	130 kDa doublet	72,000 64,000	75,000 68,000 61,000	130,000 68,000
Host range	Coleoptera	Coleoptera	Coleoptera	Lepidoptera

The cultures disclosed in this application have been deposited in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, USA.

Culture	Repository No.	Deposit date
Bacillus thuringiensis PS50C	NRRL B-18746	January 9, 1991
Escherichia coli NM522 [pMYC1638]	NRRL B-18751	January 11, 1991

B.t. PS50C, NRRL B-18746, can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle, the bacteria can be harvested by first separating the B.t. spores and crystals from the fermentation broth by means well known in the art. The recovered B.t. spores and crystals can be formulated into a wettable powder, liquid concentrate, granules, or other formulations by the addition of surfactants, dispersants, inert carriers and other components to facilitate handling and application for particular target pests. These formulation and application procedures are all well known in the art.

Plasmid DNA (pMYC1638) containing the toxin gene from B.t. PS50C can be purified from E. coli NM-522[pMYC1638] by standard procedures well known in the art. The toxin gene can be excised from the plasmid DNA by restriction enzyme digestion, as indicated in the accompanying drawing.

Formulated products can be sprayed or applied onto foliage to control phytophagous beetles or caterpillars.

Another approach that can be taken is to incorporate the spores and crystals of B.t. PS50C into bait granules containing an attractant and applying these granules to the soil for control of soil-inhabiting Coleoptera. Formulated B.t. PS50C can also be applied as a seed-coating or root treatment or total plant treatment.

The B.t. PS50C cells can be treated prior to formulation to prolong the pesticidal activity when the cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not del teriously affect the properties of the pesticide, nor diminish the cellular capability in protecting the pesticide. Examples of chemical reag nts are halog nating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be

used under mild conditions and for sufficient time to achieve th desired results. Other suitable techniques include treatm nt with aldehydes, such as formaldehyd and glutaraldehyd ; anti-infectives, such as zephiran chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Bouin's fixative and Helly's fixative (See: Humason, Gretchen. L., Animal Tissue Techniques, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of the target pest(s). Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like.

The novel toxin gene of the subject invention was obtained from a novel coleopteran-active B. thuringiensis (B.t.) isolate designated B.t. PS50C. The gene was isolated as disclosed in the Examples.

The toxin gene of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the situs of coleopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B.t. toxin.

Where the B.t. toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect hahitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes; fungi, particularly yeast, e.g., genera Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phystophère bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, and Azotobacter vinlandii; and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans. Of particular interest are the pigmented microorganisms.

A wide variety of ways are available for introducing the B.t. gene expressing the toxin into the microorganism host under conditions which allow for stable maintenance and expression of the gene. One can provide for DNA constructs which include the transcriptional and translational regulatory signals for expression of the toxin gene, the toxin gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

The transcriptional initiation signals will include a promoter and a transcriptional initiation start site. In some instances, it may be desirable to provide for regulative expression of the toxin, where expression of the toxin will only occur after release into the environment. This can be achieved with operators or a region binding to an activator or enhancers, which are capable of induction upon a change in the physical or chemical environment of the microorganisms. For example, a temperature sensitive regulatory region may be employed, where the organisms may be grown up in the laboratory without expression of a toxin, but upon release into the environment, expression would begin. Other techniques may employ a specific nutrient medium in the laboratory, which inhibit the expression of the toxin, where the nutrient medium in the environment would allow for expression of the toxin. For translational initiation, an ribosomal binding site and an initiation codon will be present.

Various manipulations may be employed for enhancing the expression of the messenger, particularly by using an active promoter, as well as by employing sequences, which enhance the stability of the messenger RNA. The initiation and translational termination region will involve stop codon(s), a terminator region, and optionally, a polyad nylation signal.

In the direct n of transcription, namely in the 5' to 3' direct n of the coding or ense sequence, the construct will involv the transcriptional regulatory regi n, if any, and the promoter, where the regulatory region

may be either 5' or 3' of the promoter, the ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the stop codon(s), the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker, where the second DNA sequence may be joined to the toxin expression construct during introduction of the DNA into the host.

By a marker is intended a structural gene which provides for selection of those hosts which have been modified or transformed. The marker will normally provide for selective advantage, for example, providing for biocide resistance, e.g., resistance to antibiotics or heavy metals; complementation, so as to provide prototropy to an auxotrophic host, or the like. Preferably, complementation is employed, so that the modified host may not only be selected, but may also be competitive in the field. One or more markers may be employed in the development of the constructs, as well as for modifying the host. The organisms may be further modified by providing for a competitive advantage against other wild-type microorganisms in the field. For example, genes expressing metal chelating agents, e.g., siderophores, may be introduced into the host along with the structural gene expressing the toxin. In this manner, the enhanced expression of a siderophore may provide for a competitive advantage for the toxin-producing host, so that it may effectively compete with the wild-type microorganisms and stably occupy a niche in the environment.

Where no functional replication system is present, the construt will also include a sequence of at least 50 basepairs (bp), preferably at least about 100 bp, and usually not more than about 1000 bp of a sequence homologous with a sequence in the host. In this way, the probability of legitimate recombination is enhanced, so that the gene will be integrated into the host and stably maintained by the host. Desirably, the toxin gene will be in close proximity to the gene providing for complementation as well as the gene providing for the competitive advantage. Therefore, in the event that a toxin gene is lost, the resulting organism will be likely to also lose the complementing gene and/or the gene providing for the competitive advantage, so that it will be unable to compete in the environment with the gene retaining the intact construct.

A large number of transcriptional regulatory regions are available from a wide variety of microorganism hosts, such as bacteria, bacteriophage, cyanobacteria, algae, fungi, and the like. Various transcriptional regulatory regions include the regions associated with the trp gene, lac gene, gal gene, the lambda left and right promoters, the tac promoter, the naturally-occurring promoters associated with the toxin gene, where functional in the host. See for example, U.S. Patent Nos. 4,332,898, 4,342,832 and 4,356,270. The termination region may be the termination region normally associated with the transcriptional initiation region or a different transcriptional initiation region, so long as the two regions are compatible and functional in the host.

Where stable episomal maintenance or integration is desired, a plasmid will be employed which has a replication system which is functional in the host. The replication system may be derived from the chromosome, an episomal element normally present in the host or a different host, or a replication system from a virus which is stable in the host. A large number of plasmids are available, such as pBR322, pACYC184, RSF1010, pRO1614, and the like. See for example, Olson et al., (1982) J. Bacteriol. 150:6069, and Bagdasarian et al., (1981) Gene 16:237, and U.S. Patent Nos. 4,356,270, 4,362,817, and 4,371,625.

The B.t gene can be introduced between the transcriptional and translational initiation region and the transcriptional and translational termination region, so as to be under the regulatory control of the initiation region. This construct will be included in a plasmid, which will include at least one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, which have been described previously. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for pesticidal activity.

Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium; Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillacea ; Pseudomonadacea , such as Pseudomonas and Acetobacter; Azotobacteraceae and

Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and th like.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducting the B.t. gene into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, stability, and the like.

Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, Streptomyces lividans, and the like.

The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

Treatment of the recombinant microbial cell can be done as disclosed infra. The treated cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of inactivation should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

The cellular host containing the B.t. insecticidal gene may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the B.t. gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

The B.t. cells may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

The pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

The formulations can be applied to the environment of the coleopteran pest(s), e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - Culturing *B.t.* PS50C, NRRL B-18746

A subculture of B.t. PS50C, NRRL B-18746 can be used to inoculate the following medium, a peptone, glucose, salts medium.

Bacto Peptone	7.5 g/l
Glucose	1.0 g/l
KH_2PO_4	3.4 g/l
K_2HPO_4	4.35 g/l
Salt Solution	5.0 ml/l
$CaCl_2$ Solution	5.0 ml/l

Salts Solution (100 ml)

$MgSO_4.7H_2O$ 2.46 g
$MnSO_4.H_2O$ 0.04 g
$ZnSO_4.7H_2O$ 0.28 g
$FeSO_4.7H_2O$ 0.40 g
$CaCl_2$ Solution (100 ml)
$CaCl_2.2H_2O$ 3.66g
pH 7.2

The salts solution and $CaCl_2$ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30°C on a rotary shaker at 200 rpm for 64 hr.

The above procedure can be readily scaled up to large fermentors by procedures well known in the art.

The B.t. spores and crystals, obtained in the above fermentation, can be isolated by procedures well known in the art. A frequently-used procedure is to subject the harvested fermentation broth to separation techniques, e.g., centrifugation.

Example 2 - Testing of *B.t.* PS50C, NRRL B-18746 Spores and Crystals

B.t. PS50C, NRRL B-18746 spores and crystals are toxic to the Colorado potato beetle (CPB). The assay for the Colorado potato beetle was conducted as follows:

CPB Bioassay - Early second instar larvae of Leptinotarsa decemlineata are placed on potato leaves which have been dipped in suspensions containing Bacillus thuringiensis preparations. The larvae are incubated at 25°C for 4 days, and larval mortality is recorded and analyzed using probit analysis.

Example 3 - Cloning of a Novel Toxin Gene from *B.t.* Isolate PS50C

Total cellular DNA was prepared from Bacillus thuringiensis (B.t.) cells grown to an optical density, at 600 nm, of 1.0. The cells were recovered by centrifugation and protoplasts were prepared in TES buffer (30 mM Tris-HCl, 10 mM EDTA 50 mM NaCl, pH = 8.0) containing 20% sucrose and 50 mg/ml lysozyme. The protoplasts were lysed by addition of SDS to a final concentration of 4%. The cellular material was precipitated overnight at 4°C in 100 mM (final concentration) neutral potassium chloride. The supernate was extracted twice with phenol/chloroform (1:1). Nucleic acids were precipitated with ethanol and DNA was purified by isopycnic banding on cesium chloride-ethidium bromide gradients.

Total cellular DNA from B.t. subsp. kumamotoensis (B.t.kum.), isolate PS50C, was digested with HindIII and fractionated by electrophoresis on a 0.8% (w/v) agarose-TAE (50 mM Tris-HCl, 20 mM NaOAc, 2.5 mM EDTA, pH = 8.0) buffered gel. A Southern blot of the gel was hybridized with a [^{32}P]-radiolabeled oligonucleotide probe. Results showed that the hybridizing fragments of PS50C are approximately 12 Kb and 1.7 Kb in size.

A library was constructed from PS50C total cellular DNA partially digested with Sau3A and size fractionated by gel electrophoresis. The 9-23 Kb region of the gel was excised and the DNA was electroeluted and then concentrated using an Elutip-d™ ion exchange column (Schleicher and Schuel, Keene, NH). The isolated Sau3A fragments were ligated into BamHI-digested LambdaGEM-11™ (PROMEGA). The packaged phage were plated on E. coli KW251 cells (PROMEGA) at a high titer and screened using the radiolabeledoligonucleotide probe. Hybridizing plaques were purified and rescreened at a lower plaque density. Single isolated, purified plaques that hybridized with the probe were used to infect E. coli KW251 cells in liquid culture for preparation of phage for DNA isolation. DNA was isolated by standard procedures. Preparative amounts of DNA were digested with XhoI (to release the inserted DNA from lambda sequences) and separated by electrophoresis on a 0.6% agarose-TAE gel. The large fragments were purified by ion exchange chromatography as above and ligated to XhoI-digested, dephosphorylated pHTBluell (an E. coli/B. thuringiensis shuttle vector comprised of pBluescript s/k [Stratagene] and the replication origin from a resident B.t. plasmid [D. Lereclus et al. 1989. FEMS Microbiology Letters 60:211-218]). The ligation mix was introduced by transformation into competent E. coli NM522 cells (ATCC 47000) and plated on LB agar containing ampicillin, isopropyl-(β)-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-4-indolyl-(β)-D-galactoside (XGAL). White colonies, with putative restriction fragment insertions in the (β)-galactosidase gene of pHTBluell, were subjected to standard rapid plasmid purification procedures. Plasmids were analyzed by XhoI digestion and agarose gel electrophoresis. The desired plasmid construct, pMYC1638, contains an approximately 12 Kb XhoI insert. A partial restriction map (see drawing) of the cloned insert indicates that the toxin gene is novel compared to the maps of other toxin genes encoding insecticidal proteins. The nucleotide sequence (open reading frame only) is shown in Sequence ID No. 1. The predicted peptide sequence of the toxin is shown in Sequence ID No. 2.

Plasmid pMYC1638 was introduced into an acrystalliferous (Cry$^-$) B.t. host (HD-1 cryB btained from A. Aronson, Purdue University) by electroporation. Expression of an approximately 130 kDa protein was verified

by SDS-PAGE. Broth containing spores and crystals was used for the determination of toxicity to Leptinotarsa decemlineata.

Plasmid pMYC1638 containing th B.t. toxin gen , can be removed from the transformed host microbe by use of standard well-known procedures. For example, E. coli NM522[pMYC1638] NRRL B-18751 can be subjected to cleared lysate isopycnic density gradient procedures, and the like, to recover pMYC1638.

Example 4 - Insertion of Toxin Gene Into Plants

The novel gene coding for the novel insecticidal toxin, as disclosed herein, can be inserted into plant cells using the Ti plasmid from Agrobacter tumefaciens. Plant cells can then be caused to regenerate into plants (Zambryski, P., Joos, H., Gentello, C., Leemans, J., Van Montague, M. and Schell, J [1983] Cell 32:1033-1043). A particularly useful vector in this regard is pEND4K (Klee, H.J., Yanofsky, M.F. and Nester, E.W. [1985] Bio/Technology 3:637-642). This plasmid can replicate both in plant cells and in bacteria and has multiple cloning sites for passenger genes. The toxin gene, for example, can be inserted into the BamHI site of pEND4K propagated in E. coli, and transformed into appropriate plant cells.

Example 5 - Cloning of Novel *B. thuringiensis* Gene Into Baculoviruses

The novel gene of the invention can be cloned into baculoviruses such as Autographa californica nuclear polyhedrosis virus (AcNPV). Plasmids can be constructed that contain the AcNPV genome cloned into a commercial cloning vector such as pUC8. The AcNPV genome is modified so that the coding region of the polyhedrin gene is removed and a unique cloning site for a passenger gene is placed directly behind the polyhedrin promoter. Examples of such vectors are pGP-B6874, described by Pennock et al. (Pennock, G.D., Shoemaker, C. and Miller, L.K. [1984] Mol. Cell. Biol. 4:399-406), and pAC380, described by Smith et al. (Smith, G.E., Summers, M.D. and Fraser, M.J. [1983] Mol Cell. Biol. 3:2156-2165). The gene coding for the novel protein toxin of the invention can be modified with BamHI linkers at appropriate regions both upstream and downstream from the coding region and inserted into the passenger site of one of the AcNPV vectors.

SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: kumamotoensis
(C) INDIVIDUAL ISOLATE: PS50C

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: LAMBDAGEM (TM) - 11 LIBRARY OF LUIS FONCERRADA
(B) CLONE: 50C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAGTCCAA ATAATCAAAA TGAATATGAA ATTATAGATG CGACACCTTC TACATCTGTA 60
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CAATTATGGC CGTCAAAGAG CGTAGATATA TGGGGAGAAA TTATGGAACG AGTGGAAGAA 360

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GATGCAAGAA CTAGAAGTGT TGTTTCTAAT CAATTTATAG CTTTAGATCT TAACTTTGTT   540
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CAGGCTGTGA ACCTACATTT ATTGTTATTA AGAGATGCTT CTATTTTTGG AGAAGAGTGG   660
GGATTTACAC CAGGTGAAAT TTCTAGATTT TATAATCGTC AAGTGCAACT TACCGCTGAA   720
TATTCAGACT ATTGTGTAAA GTGGTATAAA ATCGGCTTAG ATAAATTGAA AGGTACCACT   780
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TTGGTGGCGT TATTTCCAAA CTATGACACA CATATGTATC CAATCGAAAC AACAGCTCAA   900
CTTACACGGG ATGTGTATAC AGATCCGATA GCATTTAACA TAGTGACAAG TACTGGATTC   960
TGCAACCCTT GGTCAACCCA CAGTGGTATT CTTTTTTATG AAGTTGAAAA CAACGTAATT  1020
CGTCCGCCAC ACTTGTTTGA TATACTCAGC TCAGTAGAAA TTAATACAAG TAGAGGGGGT  1080
ATTACGTTAA ATAATGATGC ATATATAAAC TACTGGTCAG GACATACCCT AAAATATCGT  1140
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AGAATTCGCT ATGCCTCTAC AACAGATTTT GAATTTACTC TATACCTTGG CGACACAATA  1800
GAAAAAAATA GATTTAACAA AACTATGGAT AATGGGGCAT CTTTAACGTA TGAAACATTT  1860
AAATTCGCAA GTTTCATTAC TGATTTCCAA TTCAGAGAAA CACAAGATAA AATACTCCTA  1920
TCCATGGGTG ATTTTAGCTC CGGTCAAGAA GTTTATATAG ACCGAATCGA ATTCATCCCA  1980
GTAGATGAGA CATATGAGGC GGAACAAGAT TTAGAAGCGG CGAAGAAAGC AGTGAATGCC  2040
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GCGGCAAACT TAGTGGAATG CCTATCGGAT GATTTATATC CAAATGAAAA ACGATTGTTA  2160
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GAAGGGGATG CTGTATTTAA AGGACGTTAT CTACGCCTAC CAGGTGCACG AGAAATTGAT  2340
ACGGAAACGT ATCCAACGTA TCTGTATCAA AAAGTAGAGG AAGGTGTATT AAAACCATAC  2400
ACAAGATATA GACTGAGAGG GTTTGTGGGA AGTAGTCAAG GATTAGAAAT TTATACGATA  2460
CGTCACCAAA CGAATCGAAT TGTAAAGAAT GTACCAGATG ATTTATTGCC AGATGTATCT  2520
CCTGTAAACT CTGATGGCAG TATCAATCGA TGCAGCGAAC AAAAGTATGT GAATAGCCGT  2580
TTAGAAGGAG AAAACCGTTC TGGTGATGCA CATGAGTTCT CGCTCCCTAT CGATATAGGA  2640
GAGCTGGATT ACAATGAAAA TGCAGGAATA TGGGTTGGAT TTAAGATTAC GGACCCAGAG  2700
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GGATACGCAA CACTTGGAAA TCTTGAATTA GTCGAAGAGG GACCTTTGTC AGGAGACGCA   2760
TTAGAGCGCT TGCAAAGAGA AGAACAACAG TGGAAGATTC AAATGACAAG AAGACGTGAA   2820
GAGACAGATA GAAGATACAT GGCATCGAAA CAAGCGGTAG ATCGTTTATA TGCCGATTAT   2880
CAGGATCAAC AACTGAATCC TGATGTAGAG ATTACAGATC TTACTGCGGC TCAAGATCTG   2940
ATACAGTCCA TTCCTTACGT ATATAACGAA ATGTTCCCAG AAATACCAGG GATGAACTAT   3000
ACGAAGTTTA CAGAATTAAC AGATCGACTC CAACAAGCGT GGAATTTGTA TGATCAGCGA   3060
AATGCCATAC CAAATGGTGA TTTTCGAAAT GGGTTAAGTA ATTGGAATGC AACGCCTGGC   3120
GTAGAAGTAC AACAAATCAA TCATACATCT GTCCTTGTGA TTCCAAACTG GGATGAACAA   3180
GTTTCACAAC AGTTTACAGT TCAACCGAAT CAAAGATATG TATTACGAGT TACTGCAAGA   3240
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CTTACTTTTA GTGCAAGCGA TTATGATACA AATGGTGTGT ATAATGACCA AACCGGCTAT   3360
ATCACAAAAA CAGTGACATT CATCCCGTAT ACAGATCAAA TGTGGATTGA AATAAGTGAA   3420
ACAGAAGGTA CGTTCTATAT AGAAAGTGTA GAATTGATTG TAGACGTAGA G            3471
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1157 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: kumamotoensis
(C) INDIVIDUAL ISOLATE: PS50C

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Lambdagem (TM) - 11 LIBRARY OF LUIS FONCERRADA
(B) CLONE: 50C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Pro Asn Asn Gln Asn Glu Tyr Glu Ile Ile Asp Ala Thr Pro
 1               5                  10                  15

Ser Thr Ser Val Ser Ser Asp Ser Asn Arg Tyr Pro Phe Ala Asn Glu
            20                  25                  30

Pro Thr Asp Ala Leu Gln Asn Met Asn Tyr Lys Asp Tyr Leu Lys Met
        35                  40                  45

Ser Gly Gly Glu Asn Pro Glu Leu Phe Gly Asn Pro Glu Thr Phe Ile
    50                  55                  60

Ser Ser Ser Thr Ile Gln Thr Gly Ile Gly Ile Val Gly Arg Ile Leu
65                  70                  75                  80

Gly Ala Leu Gly Val Pro Phe Ala Ser Gln Ile Ala Ser Phe Tyr Ser
                85                  90                  95

Phe Ile Val Gly Gln Leu Trp Pro Ser Lys Ser Val Asp Ile Trp Gly
            100                 105                 110

Glu Ile Met Glu Arg Val Glu Glu Leu Val Asp Gln Lys Ile Glu Lys
        115                 120                 125
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Tyr Val Lys Asp Lys Ala Leu Ala Glu Leu Lys Gly Leu Gly Asn Ala
130 135 140

Leu Asp Val Tyr Xaa Gln Ser Leu Glu Asp Trp Leu Glu Asn Arg Asn
145 150 155 160

Asp Ala Arg Thr Arg Ser Val Val Ser Asn Gln Phe Ile Ala Leu Asp
165 170 175

Leu Asn Phe Val Ser Ser Ile Pro Ser Phe Ala Val Ser Gly His Glu
180 185 190

Val Leu Leu Leu Ala Val Tyr Ala Gln Ala Val Asn Leu His Leu Leu
195 200 205

Leu Leu Arg Asp Ala Ser Ile Phe Gly Glu Glu Trp Gly Phe Thr Pro
210 215 220

Gly Glu Ile Ser Arg Phe Tyr Asn Arg Gln Val Gln Leu Thr Ala Glu
225 230 235 240

Tyr Ser Asp Tyr Cys Val Lys Trp Tyr Lys Ile Gly Leu Asp Lys Leu
245 250 255

Lys Gly Thr Thr Ser Lys Ser Trp Leu Asn Tyr His Gln Phe Arg Arg
260 265 270

Glu Met Thr Leu Leu Val Leu Asp Leu Val Ala Leu Phe Pro Asn Tyr
275 280 285

Asp Thr His Met Tyr Pro Ile Glu Thr Thr Ala Gln Leu Thr Arg Asp
290 295 300

Val Tyr Thr Asp Pro Ile Ala Phe Asn Ile Val Thr Ser Thr Gly Phe
305 310 315 320

Cys Asn Pro Trp Ser Thr His Ser Gly Ile Leu Phe Tyr Glu Val Glu
325 330 335

Asn Asn Val Ile Arg Pro Pro His Leu Phe Asp Ile Leu Ser Ser Val
340 345 350

Glu Ile Asn Thr Ser Arg Gly Gly Ile Thr Leu Asn Asn Asp Ala Tyr
355 360 365

Ile Asn Tyr Trp Ser Gly His Thr Leu Lys Tyr Arg Arg Thr Ala Asp
370 375 380

Ser Thr Val Thr Tyr Thr Ala Asn Tyr Gly Arg Ile Thr Ser Glu Lys
385 390 395 400

Asn Ser Phe Ala Leu Glu Asp Arg Asp Ile Phe Glu Ile Asn Ser Thr
405 410 415

Val Ala Asn Leu Ala Asn Tyr Tyr Gln Lys Ala Tyr Gly Val Pro Gly
420 425 430

Ser Trp Phe His Met Val Lys Arg Gly Thr Ser Ser Thr Thr Ala Tyr
435 440 445

Leu Tyr Ser Lys Thr His Thr Ala Leu Gln Gly Cys Thr Gln Val Tyr
450 455 460

Glu Ser Ser Asp Glu Ile Pro Leu Asp Arg Thr Val Pro Val Ala Glu
465 470 475 480

Ser Tyr Ser His Arg Leu Ser His Ile Thr Ser His Ser Phe Ser Lys
485 490 495

Asn Gly Ser Ala Tyr Tyr Gly Ser Phe Pro Val Phe Val Trp Thr His
500 505 510

Thr Ser Ala Asp Leu Asn Asn Thr Ile Tyr Ser Asp Lys Ile Thr Gln
515 520 525

Ile Pro Ala Val Lys Gly Asp Met Leu Tyr Leu Gly Gly Ser Val Val
530 535 540

Gln Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Lys Arg Thr Asn Pro
545 550 555 560

Ser Ile Leu Gly Thr Phe Ala Val Thr Val Asn Gly Ser Leu Ser Gln
565 570 575

Arg Tyr Arg Val Arg Ile Arg Tyr Ala Ser Thr Thr Asp Phe Glu Phe
580 585 590

Thr Leu Tyr Leu Gly Asp Thr Ile Glu Lys Asn Arg Phe Asn Lys Thr
595 600 605

Met Asp Asn Gly Ala Ser Leu Thr Tyr Glu Thr Phe Lys Phe Ala Ser
610 615 620

Phe Ile Thr Asp Phe Gln Phe Arg Glu Thr Gln Asp Lys Ile Leu Leu
625 630 635 640

Ser Met Gly Asp Phe Ser Ser Gly Gln Glu Val Tyr Ile Asp Arg Ile
645 650 655

Glu Phe Ile Pro Val Asp Glu Thr Tyr Glu Ala Glu Gln Asp Leu Glu
660 665 670

Ala Ala Lys Lys Ala Val Asn Ala Leu Phe Thr Asn Thr Lys Asp Gly
675 680 685

Leu Arg Pro Gly Val Thr Asp Tyr Glu Val Asn Gln Ala Ala Asn Leu
690 695 700

Val Glu Cys Leu Ser Asp Asp Leu Tyr Pro Asn Glu Lys Arg Leu Leu
705 710 715 720

Phe Asp Ala Val Arg Glu Ala Lys Arg Leu Ser Gly Ala Arg Asn Leu
725 730 735

Leu Gln Asp Pro Asp Phe Gln Glu Ile Asn Gly Glu Asn Gly Trp Ala
740 745 750

Ala Ser Thr Gly Ile Glu Ile Val Glu Gly Asp Ala Val Phe Lys Gly
755 760 765

Arg Tyr Leu Arg Leu Pro Gly Ala Arg Glu Ile Asp Thr Glu Thr Tyr
770 775 780

Pro Thr Tyr Leu Tyr Gln Lys Val Glu Glu Gly Val Leu Lys Pro Tyr
785 790 795 800

Thr Arg Tyr Arg Leu Arg Gly Phe Val Gly Ser Ser Gln Gly Leu Glu
805 810 815

Ile Tyr Thr Ile Arg His Gln Thr Asn Arg Ile Val Lys Asn Val Pro
820 825 830

Asp Asp Leu Leu Pro Asp Val Ser Pro Val Asn Ser Asp Gly Ser Ile
835 840 845

Asn Arg Cys Ser Glu Gln Lys Tyr Val Asn Ser Arg Leu Glu Gly Glu
850 855 860

Asn Arg Ser Gly Asp Ala His Glu Phe Ser Leu Pro Ile Asp Ile Gly
865 870 875 880

Glu Leu Asp Tyr Asn Glu Asn Ala Gly Ile Trp Val Gly Phe Lys Ile
885 890 895

Thr Asp Pro Glu Gly Tyr Ala Thr Leu Gly Asn Leu Glu Leu Val Glu
900 905 910

Glu Gly Pro Leu Ser Gly Asp Ala Leu Glu Arg Leu Gln Arg Glu Glu
915 920 925

Gln Gln Trp Lys Ile Gln Met Thr Arg Arg Arg Glu Glu Thr Asp Arg
930 935 940

Arg Tyr Met Ala Ser Lys Gln Ala Val Asp Arg Leu Tyr Ala Asp Tyr
945 950 955 960

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Gln Asp Gln Gln Leu Asn Pro Asp Val Glu Ile Thr Asp Leu Thr Ala
                965                 970                 975

Ala Gln Asp Leu Ile Gln Ser Ile Pro Tyr Val Tyr Asn Leu Met Phe
            980                 985                 990

Pro Glu Ile Pro Gly Met Asn Tyr Thr Lys Phe Thr Glu Leu Thr Asp
        995                 1000                1005

Arg Leu Gln Gln Ala Trp Asn Leu Tyr Asp Gln Arg Asn Ala Ile Pro
    1010                1015                1020

Asn Gly Asp Phe Arg Asn Gly Leu Ser Asn Trp Asn Ala Thr Pro Gly
1025                1030                1035                1040

Val Glu Val Gln Gln Ile Asn His Thr Ser Val Leu Val Ile Pro Asn
                1045                1050                1055

Trp Asp Glu Gln Val Ser Gln Gln Phe Thr Val Gln Pro Asn Gln Arg
            1060                1065                1070

Tyr Val Leu Arg Val Thr Ala Arg Lys Glu Gly Val Gly Asn Gly Tyr
        1075                1080                1085

Val Ser Ile Arg Asp Gly Gly Asn Gln Ser Glu Thr Leu Thr Phe Ser
    1090                1095                1100

Ala Ser Asp Tyr Asp Thr Asn Gly Val Tyr Asn Asp Gln Thr Gly Tyr
1105                1110                1115                1120

Ile Thr Lys Thr Val Thr Phe Ile Pro Tyr Thr Asp Gln Met Trp Ile
                1125                1130                1135

Glu Ile Ser Glu Thr Glu Gly Thr Phe Tyr Ile Glu Ser Val Glu Leu
            1140                1145                1150

Ile Val Asp Val Glu
        1155
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Claims

1. A culture of a microorganism which is Bacillus thuringiensis PS50C, having the identifying characteristics of NRRL B-18746, or a mutant thereof, having activity against insect pests of the order Coleoptera.

2. A composition comprising a microorganism as defined in claim 1, or spores or crystals thereof, in association with an insecticide carrier.

3. A composition according to claim 2, which comprises beetle phagostimulants or attractants.

4. A composition according to claim 2 or claim 3, which comprises formulation ingredients applied as a seed coating.

5. A composition according to any of claims 2 to 4, in the form of a bait granule.

6. A toxin, active against coleopteran pests, having a molecular weight of c. 130 kDa and the amino-acid sequence shown in Sequence ID No. 2.

7. DNA encoding a toxin according to claim 6.

8. DNA having the nucleotide sequence shown in Sequence ID No. 1.

9. A recombinant DNA transfer vector comprising DNA according to claim 7 or claim 8.

10. A host transformed with a DNA transfer vector according to claim 9.

11. A bacterial host transformed to express a toxin according to claim 6.

12. A host according to claim 10 or claim 11, which is Escherichia coli.

13. A host according to claim 12, which is Escherichia coli NM522 (pMYC1638), as availabl from NRRL, Accession No. B-18751.

14. A host according to claim 11, which is a species of Pseudomonas, Azotobacter, Erwinia, Serratia, Klebsiella, Rhizobium, Bacillus, Streptomyces Rhodopseudomonas, Methyl phillus, Agrobacterium, Acetobacter or Alcaligenes.

15. A host according to claim 14, which is pigmented and phylloplane-adherent.

16. A host according to claim 11, which is a root-colonising bacterium.

17. Substantially intact, treated cells, having prolonged pesticidal activity when applied to the environment of a target pest, containing, intracellularly, a toxin according to claim 6.

18. Cells according to claim 17, which are prokaryotes or lower eukaryotes.

19. Cells according to claim 18, which are prokaryotic cells selected from Enterobacteriaceae, Bacillaceae, Rhizobiaceae, Spirillaceae, Lactobacillaceae, Pseudomonadaceae, Azotobacteraceae and Nitrobacteraceae, or eukaryotic cells selected from Phycomycetes, Ascomycetes and Basidiomycetes.

20. Cells according to claim 17, which are of a pigmented bacterium, yeast or fungus.

21. Cells according to claim 17, of a unicellular microorganism.

22. Cells according to claim 20, wherein the microorganism is Pseudomonas.

23. Cells according to claim 22, of Pseudomonas fluorescens.

24. Cells according to any of claims 17 to 23, which are treated with iodine.

25. The plasmid denoted pMYC1638, as available from NRRL, Accession No. B-18751.

26. A process for controlling a coleopteran insect pest, which comprises administering to the pest or the environment thereof, a microorganism as defined in claim 1, a composition according to any of claims 2 to 5, a toxin according to claim 6, a host according to any of claims 10 to 16, or cells according to any of claims 17 to 24.

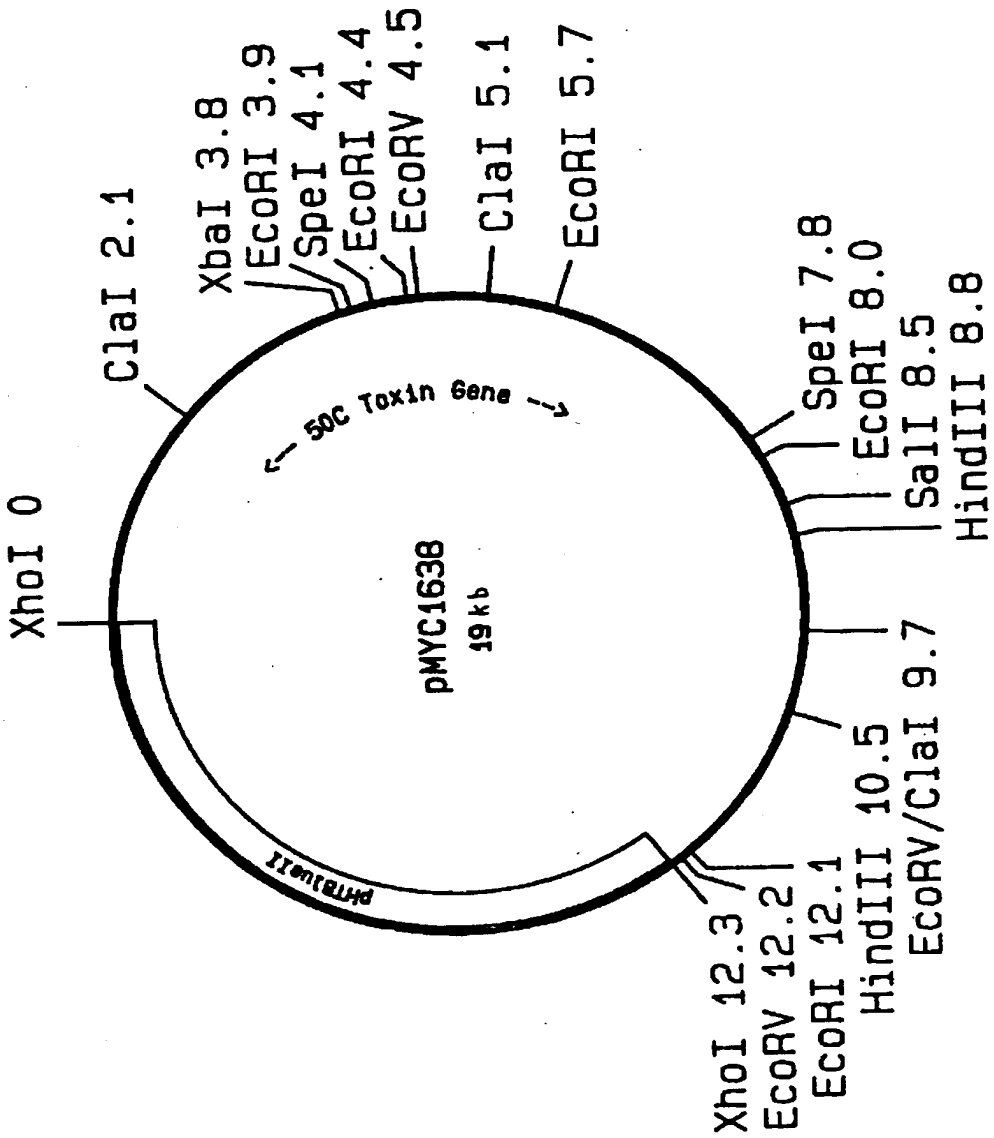
27. A process according to claim 26, wherein the pest is soil-inhabiting, which comprises applying a bait granule according to claim 5 to or into the soil.

28. A process according to claim 27, wherein the bait granule is applied at the same time corn seed is planted in the soil.

29. A process according to claim 26, wherein the pests are present on stored products.

30. A process according to claim 26, wherein the administration is to the rhizosphere, the phylloplane or a body of water.

31. A process according to any of claims 26 to 30, wherein the pest is the Colorado potato beetle.


XhoI 0
ClaI 2.1
XbaI 3.8
EcoRI 3.9
SpeI 4.1
EcoRI 4.4
EcoRV 4.5
ClaI 5.1
EcoRI 5.7
SpeI 7.8
EcoRI 8.0
SalI 8.5
HindIII 8.8
EcoRV/ClaI 9.7
HindIII 10.5
EcoRI 12.1
EcoRV 12.2
XhoI 12.3
50C Toxin Gene
pMYC1638
19 kb
pHTBlue II